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DEVELOPMENT AND VALIDATION OF A NEW, RAPID AND ECONOMICAL SURROGATE BIOASSAY FOR INDUSTRIAL CONTAMINANTS

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DEVELOPMENT AND VALIDATION OF A NEW, RAPID AND ECONOMICAL SURROGATE BIOASSAY FOR INDUSTRIAL CONTAMINANTS

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Abstract

Standard bioassays, used to determine effluent toxicity, such as static 96-hour tests using fish, and 48-hour tests using Daphnia, are primarily based on the acute lethality of toxicants to test organisms. These tests are often time-consuming, labour-intensive, or costly. This study has addressed the need for developing a new, rapid, and economical bioassay for monitoring industrial effluents. In light of the recent "battery-of-tests" approach to evaluating water quality, Environment Ontario is supporting the development of predictive dose-response relationships for aquatic organisms at all food chain levels. The specific objectives of this project were: to develop a working protocol for a behavioural toxitactic bioassay using ciliated protists; to test the protocol with standard reference toxicants and industrial effluents; and, to compare the results from this sublethal test with results from standard (lethal) tests. A working protocol was successfully established. The protocol was then applied to four standard reference toxicants: zinc, sodium chloride, 4-chlorophenol and cadmium. Sodium chloride was the only reference toxicant yielding a significant Lowest-Observed-Effect-Concentration (LOEC) value. The protocol was then applied to industrial effluents from several industries, namely, Pulp and Paper, Metal Casting, Iron and Steel, and Organic Chemical. Two standard bioassay protocols were also implemented with the same effluents. Four out of 11 effluents tested with the new protocol yielded results comparable with the two standard tests. Although these results are encouraging, the sensitivity of the test was poorer than expected, in light of results from similar studies with heavy metals (Berk *et al.* 1985). Future work on this test protocol should focus on three main areas: further experiments with reference toxicants and effluents using a pre-treatment starvation buffer which sensitizes the ciliates to toxicity; interlaboratory calibration of the protocol; and, implementing the test with other, perhaps more sensitive, species.

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1.0 INTRODUCTION

Increasing pollution of our aquatic resources has made it imperative to establish effective means of monitoring the effects of anthropogenic pollutants on aquatic biota. Although sophisticated physical and chemical techniques have been used extensively to monitor fresh-water pollutants (see review by Brezonik 1974), biological responses to pollutants have been used as indicators of toxicity only in the past few decades (Sprague 1976).

Bioassays have been applied to a number of organisms representing different trophic levels in the aquatic food chain, including fish (Sprague 1973), arthropods (Marshall 1979), algae (Wong *et al.* 1979) and bacteria (Anderson and Abdelghani 1980, Bulich *et al.* 1981). Nevertheless, the most widely-used procedure is the traditional acute bioassay, in which the lethality of various concentrations of a toxicant or toxicant mixture are measured within a predetermined exposure time. Standardized static 96-hour bioassays, such as those using fish (e.g., Craig *et al.* 1983, Environment Canada 1990, Sprague 1973), have been and continue to be used extensively by regulatory agencies (e.g., U.S. Environmental Protection Agency, Ontario Ministry of the Environment, Environment Canada) in order to determine the potential biological effects of toxic materials. Although these tests yield quantitative measures of contamination levels, they are often either time-consuming, labour-intensive, or costly. In a review article over a decade ago, Sprague (1976) suggested that tests on lower levels of the food chain (i.e., invertebrates and algae) yield a great deal of useful data, are rapid and efficient, and, most importantly, often give results comparable with traditional (lethal) bioassays using fish.

In light of these points, and the movement towards a "battery of tests" toxicological approach, there is a continuing need for the development of predictive dose-response relationships for aquatic organisms, emphasizing "lower" trophic levels. One of the main advantages of this is that organisms "lower" in the food chain may act as early warning indicators for more significant adverse effects on "higher" level food chain biota such as fish, birds, and mammals. This may be especially important where chronic toxicity is a factor.

New, rapid and economical bioassays have been developed, but their implementation and acceptance as monitoring tools has been, and continues to be, slow. For example, aquatic microbial communities have been used in the assessment of pollutant effects, although their importance and usefulness as indicators have generally been underestimated (Cairns 1974). Single species tests are more common. The Microtox^{R} test (Bulich *et al.* 1981), in which pollutant toxicity is evaluated by bacterial fluorescence, has only recently gained recognition as a standard test in the United States. Even so, some workers are reluctant to use it for tests on fresh water, since the test organism, *Photobacterium phosphoreum*, is a marine species, and the test

sample must be chemically altered prior to conducting the test. Moreover, the Microtox^{R} test is known to have variable sensitivity depending on the types of toxicants/toxicant mixtures being tested (Environment Canada 1990).

Ciliated protists (Kingdom Protista, Phylum Ciliophora) are unicellular aquatic microzooplankton which feed predominantly on bacteria (Sherr and Sherr 1987) and small phytoplankton (Bernard and Rassoulzadegan 1990, Lynn and Montagnes 1991). They are important organisms in the transfer and transformation of nutrients in both marine and freshwater food chains (Beaver and Crisman 1989, Berk *et al.* 1977, Pace and Orcutt Jr. 1981, Porter *et al.* 1985). It has been shown that energy from the microbial loop may go through ciliates, although the controversy in the literature over whether the ciliates may act as a "link" or as a "sink" is still active (Sherr and Sherr 1987).

Recently, with a movement in toxicological research towards: (a) more rapid assessment techniques, and (b) better indicators of chronic, sublethal effects of toxicants in fresh water, many investigators have turned to ciliated protists (and other aquatic microbes) as test and/or indicator organisms for the assessment of eutrophic and polluted waters, since they are sensitive to many different toxicants in the natural environment. Several comprehensive reviews in the literature provide a state-of-science perspective on this field (Curds 1982, Lynn and Gilron 1991, Nilsson 1989, Parker 1983, Persoone and Dive 1978).

This study specifically addresses the development and reliability of a new protistan bioassay technique for monitoring the toxicity of both pure compounds and industrial effluents. The sublethal bioassay technique utilizes the chemotactic responses of the ciliated protist species, *Tetrahymena vorax*, as an indicator of aquatic toxicity.

2.0 MATERIALS AND METHODS

2.1 Introduction

The approach used in this study was first to develop a protocol for a sublethal toxicity bioassay using, as an indicator, the chemotactic response of the ciliated protist, *Tetrahymena vorax*. Second, the protocol was applied to standard reference toxicants, commonly used for other standard bioassays (Environment Canada 1990). The third and final phase involved testing the protocol with toxicant mixtures (effluents) from several industries, namely, Pulp and Paper, Metal Casting, Iron and Steel, and Organic Chemical. During this final phase, two other standard OMOE bioassay protocols (*Daphnia magna* 48-hour acute lethality test, Poirier *et al.* 1988; rainbow trout 96-hour acute lethality test, Craig *et al.* 1983) were also implemented using the same effluents. This allowed for comparison between the ciliate protocol and other standard assays, in order to understand the relationships between the respective bioassays and to determine the utility and feasibility of the ciliate protocol, in light of standard test results.

2.2 The T-Maze Toxitactic Assay (TMTA): Protocol Development

The bioassay technique developed and validated in this study combined a behavioural chemotactic T-maze assay approach modified from a protocol developed by Van Houten *et al.* (1975, 1982) and a toxicological approach taken by Berk and colleagues (Berk *et al.* 1985, 1990, Roberts and Berk 1990). The assay is a sublethal test that measures the chemotactic behavioural response exhibited by ciliated protists (in this case, *Tetrahymena vorax*), when they encounter noxious (or toxic) chemical stimuli (hence, "toxi"-tactic). This response is quantitatively evaluated as an index based on the proportion of the test population responding to the toxicant mixture (see 2.2.2 below).

2.2.1 The Test Organism, Tetrahymena vorax, and Culture Protocol

A description of the general biology of *Tetrahymena* is available in Nilsson (1989). Furthermore, a thorough description of the general biology of *Tetrahymena vorax*, a common protistan laboratory organism, is available in Lawrence *et al.* (1981).

In the first phase of the project, cultures of *Tetrahymena vorax*, the test organism used in the assay and in the study, were established, and were maintained successfully since their initiation. The following is the culture protocol used for maintaining these cultures:

A culture of *Tetrahymena vorax* Kidder, 1941 (Strain V2S) was obtained from Dr. N.E. Williams, University of Iowa.

A Proteose Peptone Yeast Extract (PPYE) medium was prepared in the following proportions (depending on culture medium requirements):

Distilled Water	400.0 ml
Dextrose	0.8 g
Proteose Peptone	2.0 g
Yeast Extract	2.0 g

The distilled water was heated in a beaker over a Bunsen burner. Dextrose was then added and the solution was stirred. The proteose peptone was added and then mixed in. The yeast extract was added, but not stirred in. The solution was then heated until the yeast extract was dissolved, but not allowed to boil. Ten ml aliquots were then dispensed into culture tubes. The tubes were capped, and autoclaved for 20 minutes at 15 p.s.i. Using sterile techniques, the culture was then transferred every two weeks.

2.2.2 Test Protocol Description

In the first phase of the project, a protocol for the bioassay was developed. It has been called (and is heretofore referred to as) the T-Maze Toxitactic Assay (TMTA).

The following protocol is carried out for each T-maze (i.e., one T-maze run represents one concentration replicate):

1. All solutions to be used in the assay must be at room temperature (20°C ± 2°C).

2. Twenty ml (sometimes more, depending on cell density) of a *Tetrahymena vorax* culture (48-hour-old cell culture; see 2.2.3 below) are centrifuged in a conical 12-15 ml tube at 1200 rpm for 3 minutes. With a pipette, the pellet of concentrated cells is then removed, ensuring that the least amount of culture fluid is taken with the pellet.

3. With a pipette, the pellet is then added to a second centrifuge tube, resuspended in dilution water, and the level brought even to that in another tube with dilution water[1]. The suspension is then re-centrifuged, the pellet is removed again, and is re-suspended once again before adjusting the density. Cell density is then adjusted to approximately 200,000 cells/ml.

[1] Dilution water refers both to the control solution and the substance used to dilute the stock solutions. It was produced by diluting well water to a conductivity of 300 ± 5 μmhos with reverse osmosis water. A complete table describing the physico-chemical properties of the dilution water can be found in Appendix II.

4. The T-maze apparatus is represented in Figure 1 (see Appendix I for a list of apparatus parts and catalogue numbers). The stopcock is turned so that the bore is in line with the third (upright) arm. The test arms of the T-maze apparatus are then filled, one at a time, with the respective solutions (one control, one test). Small Pasteur pipettes are used to deliver the solutions into the arms to prevent air from being caught in the arms of the T-maze. Each arm is then corked (the corks used should have been soaked in dilution water for 24-48 hours before use). It must then be ensured that no air bubbles are caught in the arms, particularly around the stopcock. Holding one arm upwards at a 45° angle, all air bubbles are shaken out by firmly hitting the T-maze apparatus on the palm of the hand. This is repeated for the second arm. Re-corking should be done, if necessary, to release any air bubbles.

5. With a very long Pasteur pipette, the cells are then transferred from a homogeneous suspension into the stopcock barrel (the solution is filled above the level of the bore). The bottom of the apparatus is then tapped gently (to remove any initial air bubbles).

   NOTE: removal of the air bubbles is crucial to running the assay properly, since they will prevent organisms from migrating into the arms.

6. After the T-maze is filled completely, the test can then begin. The stopcock is turned so that the cells are able to migrate freely through the arms. It is critical that the bore be completely level at this point. At the end of the 15-minute exposure period, the stopcock is then turned again to stop the assay.

7. Immediately after the assay is completed, the arms of the T-maze are emptied into counting tubes (this includes a thorough rinse with test water from each arm to ensure that all cells have been removed). Lugol's iodine solution (~0.05 ml) is then added to each tube and each tube is mixed. All tubes are covered with Parafilm^{R} until they are ready to count. Before enumeration, the T-mazes must immediately be rinsed thoroughly with tap water, then twice with distilled water, in preparation for acid washing.

8. The cells are now ready to enumerate: the counting tubes are homogenized by inverting the tubes or by using an automatic vortex machine. One twenty-fifth of a ml (0.04 ml) is pipetted out into a Palmer counting cell (see Appendix I). All cells in the counting chamber are enumerated. No fewer than two aliquots are counted per counting tube (if the two estimates are out by more than 10 %, another aliquot is counted). If necessary, a smaller aliquot may be counted if the cells are too dense. As a guideline, the densest arm (where accumulation/attraction has occurred) should have at least 2500 cells/ml. The replicate counts are recorded on a standard data sheet (see Appendix III).

Calculation of the Toxitactic index (I_{tox})

A "toxitactic" index (I_{tox}) is then calculated (for each T-maze) as follows:

$$I_{tox} = \frac{\text{\# of cells in test arm (T)}}{\text{(\# of cells in test arm (T) + \# of cells in control arm (C))}}$$

OR (abbreviated)

$$I_{tox} = \frac{T}{T + C}$$

(The index is therefore a number between 0 and 1)

Determination of the Lowest-Observed-Effect-Concentration (LOEC) value

The lowest concentration of a toxicant to which the ciliates are exposed in the assay which causes a statistically significant response, is referred to as the lowest-observed-effect-concentration (LOEC) value.

The LOEC is value determined as follows:

(i) The I_{tox} values for the concentrations tested and a control (y-axis) are plotted against concentration of the toxicant (x-axis).

(ii) When there is a response, the trend is either:

case (a) increasing I_{tox} with increasing concentration (attraction); or, case (b) decreasing I_{tox} with increasing concentration (repulsion).

(iii) An analysis of variance (ANOVA; see Snedecor and Cochran 1967) and a multiple range test (e.g., Tukey's multiple range comparison) are performed on all of the data for a given experiment, to determine the lowest concentration at which the I_{tox} value is (statistically) significantly different from the control I_{tox}.

(iv) The concentration at which this occurs is the LOEC value.

2.2.3 Protocol Standardization Tests

Throughout the establishment of the protocol, several "standardization" tests/activities were conducted. These were intended to ensure the precision and rigour of the methodology. The design and implementation of these tests/activities is briefly described below.

T-maze Rack Design

In order to ensure that the migration of cells was not influenced by gravity (since ciliates are negatively geotropic), a wooden T-maze rack (based on a design by Dr. J. Van Houten, pers. comm.) was constructed and carefully levelled. This rack was used to hold the T-mazes during all experiments.

Clonal Age Tests

Ciliated protists follow a classical microbial growth curve, with a lag phase, an exponential phase, and a stationary phase (Fenchel 1987). The exponential phase is that part of the growth curve in which there is the most rapid increase in growth, while the stationary phase is characterised by much slower growth. This slower growth may be associated with physiological changes that may affect the behaviour of cells in a population. For this reason, and the fact that many other studies use exponential phase cells in implementing standard techniques (e.g., Houba *et al.* 1981), it was decided that only cells in the exponential phase of growth be used in this behavioural assay. In several experimental cultures, it was determined that cells between 24 and 48 hours old were in the exponential phase of population growth. Cells in this phase were routinely used for the TMTA assay and all other experiments.

Control Tests

In order to ensure that results with test toxicants could be properly compared, experiments using only control solutions (i.e., no toxicants) were implemented. This, in "chemotactic" theory, should produce equal numbers of cells from the test population in both arms of the T-maze (i.e., yielding an I_{tox} value = 0.5) and would ensure that there are no adverse maze effects. All T-mazes were tested for this.

Cell Enumeration Precision

Throughout the implementation of all experimental test runs, running totals of all replicate counts for each run were recorded in order to evaluate the precision associated with cell enumeration subsequent to an experiment. This precision is crucial to the statistical integrity of the test, since cell counts are used to calculate the I_{tox} value, the effective indicator for the test.

NOTE: Each test series (all concentrations, all replicates) took 3-3 1/2 hours to enumerate.

2.3 Reference Toxicant Tests

2.3.1 Preparation of Reference Toxicant Solutions

Reference toxicant solutions were prepared as follows (all aliquots of all solutions were weighed to three decimal places on a top-loading electronic balance (except for cadmium, see below)):

Zinc

The concentrations of zinc sulphate used in testing were prepared from the stock solutions also used for rainbow trout tests (Craig *et al.* 1983). These included 3200, 1600, 800, 400 and 200 μg l^{-1} of $ZnSO_4 7H_2O$. To prepare the dilutions used in the TMTA test, 5 ml of each of the above were diluted to 500 ml in a volumetric flask with dilution water. The zinc in the original stock solution was in a concentration of 3200 μg l^{-1}.

Sodium Chloride

A stock solution of 5000 mg l^{-1} of sodium chloride was used. Five grams were diluted to 1000 ml in a volumetric flask with dilution water. To obtain the concentrations used for testing, an aliquot of the last solution produced was diluted by 50 % until 5 concentrations, including 5000 mg l^{-1}, were obtained.

4-chlorophenol

Stock solutions of 4-chlorophenol were not always of the same volume and/or concentration. However, the same basic method was used for the preparation of each. The chemical was diluted in a volumetric flask using dilution water. Dilutions were made using graduated cylinders. In most cases, the dilution series began with 150 mg l^{-1} and the solutions were serially diluted by 50 % until 5 concentrations were obtained.

Cadmium

One litre of a stock solution of 18067 ppb Cd, (in the form $Cd(NO_3)_2.4H_2O$) was produced (made in reverse osmosis water). The cadmium was weighed to five decimal places on an analytical balance. A 55.35 ml aliquot was diluted to 1.0 l with dilution water to yield a concentration of 1000 ppb. This solution was used in the first experimental run. For the other two experimental runs, 55.35 ml were diluted to 2.0 l in a volumetric flask to yield 500 ppb. Dilutions for the test were prepared in 500 ml volumetric flasks.

2.3.2 Implementation of Reference Toxicant Tests

Reference toxicants were chosen in conjunction with the Project Liaison Officer, as per requirements set out by the Ontario Ministry of the Environment. Reference toxicant tests were implemented with the TMTA using the protocol described in section 2.2.2 (Test Protocol Description), for two important reasons:

(i) to determine estimates of concentrations with which tests should be run for given classes of toxicants (e.g., heavy metals, organics); and,

(ii) to compare these results with those of other standard bioassays using similar reference toxicants.

It was decided that these tests would be carried out with four different reference toxicants, commonly used for similar purposes for other toxicity bioassays (Environment Canada 1990). The reference toxicants tested in this phase of the project were: zinc (as zinc sulphate), 4-chlorophenol, sodium chloride, and cadmium (as cadmium nitrate). The first three reference toxicants tested were chosen for comparability to data from bioassays with other taxa (i.e., *Daphnia magna*, rainbow trout tests), while the fourth toxicant, cadmium, was tested for comparability to data from another ciliate protocol using chemotaxis as the test parameter (Berk *et al.* 1985).

2.3.3 Tetrahymena vorax Microplate 24-hour LC_{50} Tests with Zinc

In addition to the TMTA (sublethal) tests, a simple 24-hour *Tetrahymena vorax* acute lethality (LC_{50}) test using a 96-well plastic microplate (used commonly for microalgal tests), was devised and tested with zinc. This test was implemented using three different preparations, as described below.

Preparation of the Dilution Series

A stock solution of 180,000 ppb Zn was used. In its production, $ZnSO_4 \cdot 7H_2O$ was dissolved in reverse osmosis water in a volumetric flask. A serial dilution of 0, 6.25, 12.5, 25, 50 and 100 % was made by diluting the stock solution with laboratory dilution water.

Preparation of the Microplate Apparatus

Microplates (Falcon #3912 (Microtest III flexible assay plate)), each consisting of 96 wells in 12 columns of 8, were rinsed in 2 % nitric acid for a short period of time. They were then rinsed in tap water, followed by a rinse with either reverse osmosis or deionized water. They were dried at moderate temperature in a drying oven.

Ciliate Bioassay Development

Two hundred μl of the five test concentrations, as well as a control, were each placed separately into three columns of wells. At this point, the plates were ready for addition of the ciliates.

Ciliate Preparations

In each of the three test runs, ciliates were prepared in three different ways:

Preparation 1

The ciliates were centrifuged out of the PPYE growth medium, suspended in dilution water and then re-centrifuged. The pellet of cells was then removed and diluted in a petri dish with dilution water. One microplate was used to adjust cell density. Sixteen wells (at a time) were filled with 200 μl of dilution water. Ten μl of the cell suspension was added to each of the wells, followed by one drop of Lugol's iodine solution. The cells were allowed to settle for five to ten minutes, after which time they were enumerated. This procedure was repeated until the density was sufficiently diluted so that approximately 10 cells were visible in each well. This was the final density used in the test.

Preparation 2

The ciliates were centrifuged as in Preparation 1, but the final suspension was left in a test tube in dilution water for approximately six hours before diluting to the appropriate concentrations as above. The purpose of the waiting period was to allow for any cell divisions in progress to be completed prior to use in the test.

Preparation 3

The ciliates were centrifuged as before, but this time they were washed with a MOPS buffered salts solution (Roberts and Berk 1990) and left for approximately six hours before testing. The switch from dilution water to the MOPS solution reduced the high mortality that resulted following the procedure in Preparation 2.

Implementation of the Test

Ten μl of the ciliate suspension were added to each of the 144 wells. An octapet (Nichiryo, #8800) was used to deliver to 8 wells (one entire column) at a time. Therefore, the pipette was only filled once. Immediately following addition of the suspension, one drop of Lugol's iodine solution was added randomly to four wells of each column (or one-half of the wells at each concentration). The purpose of this was to preserve the cells at $t=0$ in order to estimate the initial cell density in each well.

The microplates were then covered and left for an exposure period of 24 hours. Prior to the exposure period, the remaining wells then received an addition of Lugol's iodine solution. The plates were left for at least a half hour prior to enumeration. The number of cells preserved in all wells was determined and recorded.

Calculations

The mean number of cells and the standard deviation for all wells at t=0 (n=72) was calculated. The means and standard deviations were calculated for each concentration at t=24 hours (n=12). The percent mortality at each concentration was calculated as follows:

$$\text{Percent Mortality} = \frac{\bar{X}_{t=0} - \bar{X}^{c}_{t=24}}{\bar{X}_{t=0}} \times 100$$

Where: $\bar{X}_{t=0}$ is the mean of all values obtained for time = 0 hours.

$\bar{X}^{c}_{t=24}$ is the mean, at 24 hours, of all wells at a given concentration.

2.3.4 TMTA Tests Using a Starvation Buffer

In addition to the TMTA and Microplate LC_{50} tests described above, several TMTA tests using a starvation buffer were also implemented with two of the reference toxicants (sodium chloride and zinc). The major intention of this was to determine if preparing the ciliates (in a MOPS starvation buffer medium for a period of 18 hours prior to the implementation of the test; Roberts and Berk 1990) would affect the responses observed in the test (i.e., would the starvation buffer sensitize the ciliates to the toxicant ?).

2.4 Effluent Tests

In the third phase of the project, effluent tests with the TMTA protocol were carried out. It was decided in conjunction with the Project Liaison Officer that these effluent tests would be implemented with effluents from four different industrial sectors, namely: Pulp and Paper, Metal Casting, Iron and Steel, and Organic Chemical.

2.4.1 Preparation of Effluent Sample Solutions

Each effluent tested using this assay was collected from a composite including all pails of that sample which arrived from the industry at B.A.R. Environmental. The composite was well mixed at a temperature close to 15°C. Three separate 500 ml samples[1] were collected in Erlenmeyer flasks as well as a fourth in a 50 ml Erlenmeyer flask. The flasks were then immersed in hot water until the sample temperature rose to 20°C.

The smaller sample was used in a 15-minute spot test in order to determine the dilution of the original sample to be used as the highest concentration in the TMTA assay. First, samples of 100 % effluent were placed into each well of a triple-spot depression. The same was done with dilution (control) water. A single Pasteur pipet drop of *Tetrahymena vorax* cells (density approx. 200,000 cells/ml) was added to each depression. The cells were then observed for a 15-minute period. If cell movement ceased in the effluent sample as compared to the control, the test was repeated at a lower concentration. This technique was repeated until reaching a concentration that did not render the organisms immobile. The highest concentration found to be non-immobilizing to $\geq$ 80% of the cells was used as the initiation point for the dilution series used in the assay.

Five hundred ml of effluent were needed for each test replicate to be run. This was necessary to produce the various effluent concentrations required in the test. If dilution of the original sample was found to be unnecessary, the concentrations were produced from the effluent at full strength. However, if the effluent was found to be lethal, the three samples were diluted to produce three 500 ml aliquots of the appropriate concentration as indicated by the spot test. The test dilutions were then completed as though the highest concentration used was 100 % effluent (see below). The actual concentrations were calculated and recorded on the standard TMTA bench sheet (Appendix III).

Two hundred and fifty ml of a series of five effluent concentrations plus a control were prepared for each replicate. Dilutions were as follows:

Concentration %	Volume of Effluent (ml)	Volume of Dilution Water (ml)
0	0.0	250.0
6	15.0	235.0
13	32.5	217.5
25	62.5	187.5
50	125.0	125.0
100	250.0	0.0

[1] There were two exceptions. Sample numbers 03910155 and 03910178 were collected in 2 l Erlenmeyers and subsequently divided into three 500 ml samples before performing the dilutions.

All effluent volumes were measured and diluted in a 250 ml glass graduated cylinder with the exception of the 6 % solution. In this case, the effluent was measured in a 25 ml graduate and transferred to a 250 ml graduate where it was diluted. Approximately 20 ml of each dilution were transferred to labelled 50 ml beakers. These samples were introduced to the mazes in the assay. The remainder were deposited into plastic jars for analysis of dissolved oxygen, pH and conductivity.

The dilutions were performed separately for each of the three original samples so that the solutions used for each test replicate were independent of the others.

2.4.2 Implementation of Effluent Tests

For each effluent sample tested in this phase of the project, a split sample was used to run the TMTA protocol using at least five concentrations and a control (with replication). The rest of the sample was used to carry out standard OMOE protocols with *Daphnia magna* (Poirier *et al.* 1988) and rainbow trout (Craig *et al.* 1983). The TMTA test was always run concurrently with these two other tests.

2.5 Tabulation of Data

For the TMTA protocol, data were tabulated on a standard bench sheet developed specifically for this project (see Appendix III). For the *Daphnia magna* and rainbow trout tests, data were tabulated using standard bench sheets already being used by B.A.R. Environmental. For the *Tetrahymena vorax* Microplate 24-hour LC_{50} Test with zinc, data were tabulated on another bench sheet developed specifically for the test (see Appendix IV).

2.6 Data Analyses

2.6.1 Statistical Analyses

Statistical analyses were performed on all of the data. These included basic parametric statistics (means, standard deviations, etc.) and analysis of variance where appropriate. Snedecor and Cochran (1967) was used as a reference when required.

2.6.2 Computer Analyses

From the standard bench sheets, all data were entered into a microcomputer system for all analyses. LC_{50} and EC_{50} values were calculated using specialized software: PROBIT (Hubert 1987, Stephan 1977), while other data were analysed with LOTUS 1-2-3 (Lotus Development Corportion, USA).

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3.0 RESULTS AND DISCUSSION

3.1 Protocol Standardization Tests

T-Maze Rack Design

Based on the results of control tests (see below) and continual checks on the level of the mazes and the rack, it was evident that the design and use of the T-maze rack was successful in eliminating biases on the test due to gravity. This eliminates a potentially important source of error for the test, as indicated by other applications of this methodology (Antipa *et al.* 1983, Van Houten *et al.* 1982).

Control Tests

A summary of the results for all control tests are presented in Table 1 and Fig. 2. The mean I_{tox} value for all control maze runs (n=56) was 0.50 with a mean coefficient of variation (CV) (for replicates of the same maze) of 14.07 % (Table 2). In cases where there was more than one "control" run for a given maze, the data were compared for that maze (i.e., a CV was determined), in order to also determine intramaze variability. These data are represented in tabular form (Table 2) and graphically in Fig. 3.

The mean coefficient of variation (CV = 14.07 %) was compared to similar data with a chemotactic T-maze assay study, implemented by Antipa *et al.* (1983) with other ciliate species: *Paramecium caudatum, P. octaurelia* and *Didinium nasutum*. Their (Antipa *et al.* 1983) coefficients of variation using these species, ranged from 11.82 % to 15.96 % (n ranged from 6 to 34) with a mean CV of 15.31 %. These values are in the same range as our coefficient of variation (cf. 14.07 %).

Cell Enumeration Precision

The coefficients of variation (CV) for replicate counts of each of the 572 experimental runs were calculated and the results are presented graphically in Figure 4. The mean CV for all runs was 20.6 %.

3.2 Reference Toxicant Tests

A summary of test runs performed with reference toxicants is presented in Table 3. Test results for each of the reference toxicants are presented in Figures 5 to 8 and are discussed briefly below, separately.

3.2.1 Zinc

The results for zinc (in the form of zinc sulphate), based on 4 experimental runs, representing 10 different concentrations between 0 and 8 mg l^{-1} $ZnSO_4$, are presented graphically in Fig. 5. Although there is some scatter about the theoretical "no response" I_{tox} line of 0.5, especially below 2 mg l^{-1}, there appears to be an absence of a toxitactic response to zinc using this test. These results are not consistent with other toxicological tests in which ciliates were used. Slabbert and Morgan (1982), using oxygen uptake as an indicator, and *Tetrahymena pyriformis* as the test organism, reported a minimum concentration of 0.1-0.5 mg l^{-1} affecting oxygen uptake rate (Table 4). In another ciliate bioassay, using growth inhibition as an indicator, and *Colpidium campylum* as the test organism, Dive and Leclerc (1975) reported a Minimum Active Dose (MAD) of 100 mg l^{-1} for zinc (Table 4). Moreover, tests with *Daphnia magna* and rainbow trout also show sensitivity to zinc in a similar range (Tables 5, 6).

3.2.2 Sodium Chloride

The results for sodium chloride, based on 11 experimental runs, representing 6 different concentrations between 0 and 10000 mg l^{-1} NaCl, are presented graphically in Fig. 7. Of all reference toxicants tested, sodium chloride gave the most promising results, yielding an LOEC value of 2500 mg l^{-1} (see Fig. 7). The general trend of response for this toxicant was increasing I_{tox} with increasing concentration (i.e., attraction to the chemical), although a threshold of response (attraction vs. repulsion) can possibly be operating below 1000 mg l^{-1}. The LOEC value reported here is in a similar range to EC_{50} data reported for *Daphnia magna* (Table 5). Keating and Dagbuson (1986) reported an EC_{50} range for *Daphnia magna* of 2250-4500 mg l^{-1}, while Cowgill (1987) reported results in a similar range of 1661-4571 mg l^{-1}. Other unpublished data from the Ontario Ministry of the Environment and B.A.R. Environmental are also in a similar range (see Table 5).

3.2.3 4-chlorophenol

The results for 4-chlorophenol, a common organic narcotic agent, based on 7 experimental runs, representing 11 different concentrations between 0 and 150 mg l^{-1} 4-chlorophenol, are presented graphically in Fig. 6. Again, although there is some scatter about the theoretical "no response" I_{tox} line of 0.5 throughout the spectrum of these concentrations, there is no consistent trend in toxitactic response to 4-chlorophenol using this test. There are presently no results from other ciliate bioassays with which to compare this lack of response. The fact that there was no response using the TMTA test, though, is not surprising. The compound 4-chlorophenol is a narcotic agent, and affects organisms with cells in tissues (i.e., affects the transmission of impulses from one cell to another). As a result, one would not predict a taxis response from a single-celled organism. Results using other organisms are not very common, since 4-chlorophenol has only been used recently in Canadian

toxicological laboratories (Environment Canada 1990). Nevertheless, some recent data has yielded LC_{50}'s for *Daphnia magna*: 3.82 mg l^{-1} (B.A.R. Environmental, unpubl. data) and a range of 2.5 - 7.3 mg l^{-1} (OMOE, unpubl. data) (see Table 5). In addition, some recent unpublished results with rainbow trout indicate very low LC_{50}'s in the range 0.15-0.18 mg l^{-1} (OMOE, unpubl. data).

3.2.4 Cadmium

The results for cadmium (in the form of cadmium nitrate), based on 3 experimental runs, representing 6 different concentrations between 0 and 0.5 mg l^{-1} $Cd(NO_3)_2$, are presented graphically in Fig. 8. For cadmium there was little scatter about the "no response" I_{tox} line, hence, a toxitactic response to cadmium using this test was also absent. These results are not consistent with several other toxicological tests in which ciliates were used. Using an oxygen uptake rate bioassay with *Tetrahymena pyriformis* as the test organism, Slabbert and Morgan (1982) reported a minimum concentration of 0.1-0.5 mg l^{-1} affecting oxygen uptake rate (Table 4). In two other ciliate bioassays using growth inhibition as an indicator, and *Colpidium campylum* and *Tetrahymena pyriformis* as the test organisms, respectively, Dive and Leclerc (1975), reported a Minimum Active Dose (MAD) of 0.2 mg l^{-1} for cadmium, while Houba *et al.* (1981) reported an EC_{50} of 2.6 mg l^{-1} (Table 4). In other chemotactic response bioassays with *Tetrahymena pyriformis*, Berk *et al.* (1985) reported an EC_{50} range of 0.35-0.70 mg l^{-1} Cd, and Roberts and Berk (1990) reported an LC_{50} of 0.092 mg l^{-1} Cd. Moreover, tests with *Daphnia magna* and rainbow trout also indicate cadmium toxicity in a similar range to these other ciliate bioassays (see Tables 5, 6).

3.2.5 Tetrahymena vorax Microplate LC_{50} Tests with Zinc

A summary of all data from the microplate LC_{50} tests with zinc is presented in tabular form in Table 7 and graphically in Figure 9. From these results, we were only able to calculate an LC_{25} of 0.16 mg l^{-1} for zinc using this test, using Preparation 3. These tests, representing three different preparations (see section 2.2.3 above) indicate that Preparation 3 (in which the ciliates were centrifuged but were washed with a MOPS buffered salts solution and left for six hours before testing) yielded the best results (i.e., a consistent trend of response with increasing concentration), and lend support to the use of the MOPS starvation buffer as a way of sensitizing the ciliates to the TMTA test.

3.2.6 TMTA Tests Using a Starvation Buffer

TMTA tests were also implemented with two of the reference toxicants (sodium chloride and zinc), except that in these tests, the ciliates were pre-treated with a MOPS starvation buffer medium for a period of 18 hours prior to the implementation of the test (Roberts and Berk 1990). During these tests, two other species

(*Tetrahymena thermophila* and *Glaucoma chattoni*) were also used in order to assess differential species sensitivity with the TMTA protocol.

The results of these tests are represented in Figures 10, 11, and 12. Although the ciliates were more responsive (i.e., more sensitive) during the test, with pre-treatment (as indicated by Roberts and Berk (1990)), the results do not appear to yield consistent trends across species or across reference toxicants. Nevertheless, it is interesting to note that for sodium chloride, the trend of the response for *T. thermophila* is similar to that observed for *T. vorax* when cells were not pre-treated (see Figure 7). The fact that the cells appear to be more responsive, is, in itself, a good reason to pursue further study with this pre-treatment technique (see 4.2 Recommendations).

3.3 Effluent Tests

A summary of all test runs performed with effluent mixtures, indicating the number of effluents tested and the number of experimental runs per effluent is presented in Table 3. The results for each effluent experimental run using the TMTA test are represented graphically in Figures 13 through 23. Finally, Table 8 shows a comparison of the results for all three bioassays which were run concurrently.

Although the results are quite variable, there are some interesting comparisons to be made (Table 8). For example, for pulp and paper sample # 03910104, the results for the LOEC for the TMTA test and the LC_{50} for the *Daphnia magna* test were in a similar range (LOEC = 50.0 % for TMTA vs. LC_{50} = 35.8 % for *Daphnia magna* acute lethality test). In two other cases with pulp and paper effluents (effluent samples #03910110 and #03910114), the TMTA test showed no effect response, while the rainbow trout test showed a non-lethal response. For the former of those two tests (#03910110), the *Daphnia magna* test yielded an LC_{50} of > 100 %, indicated a similar result for all three tests.

On the other hand, there were also some very dissimilar results. For example, metal casting sector sample #03910095 showed a no effect response in the TMTA test indicating low toxicity, yet yielded some very low LC_{50} results with both the *Daphnia magna* and trout tests, indicating extremely high toxicity.

4.0 CONCLUSIONS AND RECOMMENDATIONS

4.1 Conclusions

Results from many recent toxicological studies with ciliated protists used as test organisms have already demonstrated that these protists can be effective bioassay organisms (Berk *et al.* 1985, 1990; Dive and Leclerc 1975; Houba *et al.* 1981; Slabbert and Morgan 1982). Nonetheless, most of these studies have used single toxicants in their evaluation of toxicity. To our knowledge, this is the first study of its kind testing whole effluents (with mixtures of potential toxicants) and with ciliates as the test organisms. It is not surprising, then, that there are problems and issues that arose in this study which require further resolution.

4.1.1 Protocol Development

The goal of the first phase of the project was to establish cultures of the test organism, *Tetrahymena vorax* and to develop a working protocol with which reference toxicants and effluents could be tested, in order to evaluate the utility and feasibility of the TMTA protocol.

Cultures of the test organism were established successfully and maintained throughout the duration of the project. The protocol development phase of the project yielded very positive results, as indicated by: tests with the T-maze rack, designed to eliminate biases due to gravity; clonal age tests, implemented in order to determine the age of cells in exponential phase of growth; and, control tests, designed to determine the precision and rigour of the test itself.

The first aspect of the protocol that requires refinement is the treatment of cells prior to the initiation of the test. The MOPS starvation buffer medium appears to sensitize the cells to reference toxicants. This is consistent with the suggestion of Roberts and Berk (1990) who discovered that this pre-treatment of the cells was necessary for consistent and comparable results.

A second aspect of the protocol that may be modified relates to the use of different species as test organisms. The test organism used in this study, *Tetrahymena vorax* has not been used in many other toxicological studies. It is possible that other, more widely-used laboratory species (such as *T. pyriformis* and *Colpidium campylum*) may have better responses. Some of our preliminary results with other ciliate species support this (see 3.2.6).

4.1.2 Reference Toxicant Tests

Tests were implemented with four reference toxicants. Using the TMTA protocol, the only reference toxicant that yielded a significant LOEC value was sodium chloride.

In this preliminary testing, it appears that the test, implemented with zinc and cadmium (4-chlorophenol is a narcotic agent and was not expected to yield significant results), is not sufficiently sensitive (in relation to other standard tests), although pre-treatment of cells through the use of a starvation buffer may yield more promising results (see above).

Previous ciliate toxicity data using chemotaxis as the test parameter, and heavy metals as test toxicants (Berk *et al.* 1985) indicates that ciliates may respond sublethally in a similar range as *Daphnia* and trout do in acute lethality tests. Results from other ciliate toxicity tests are based on slightly longer response times (i.e., growth rate) or utilize direct physiological parameters (i.e., respiration) and so are not strictly comparable with this protocol, since it utilizes a test parameter that relies on a relatively rapid behavioural response.

4.1.3 Effluent Tests

TMTA tests were implemented with effluents from several industrial sectors. Using the TMTA test, the majority of effluents had little effect on the response of the ciliates. Only 4 out of 11 tests yielded results comparable to results from acute lethality tests. Interestingly, these similarities were observed with effluents from the pulp and paper sector. It is possible, therefore, that the test, subsequent to further refinement, may prove useful specifically for pulp and paper effluents.

There are several reasons why results from tests using whole effluents may be problematic: first, a given effluent is made up of more than one toxicant, often acting in a complex series of relationships (that have only recently begun to be understood); and, second, effluents are not consistent from one sampling to another. Since there are no comparable data published that used ciliates as test organisms, and whole effluents as test toxicants, it is difficult to evaluate the potential success of this test protocol without further study.

4.2 Recommendations

A number of important issues relating to future research on the development of this bioassay protocol emerged from the results of this study. Our recommendations are presented here for consideration by the Ontario Ministry of the Environment.

Further experimental work in developing this bioassay protocol should be continued. A number important refinements to the protocol should be considered:

A. Exploration of the variation in the preparation of cells used in the test protocol:

Roberts and Berk (1990) demonstrated that an 18-hr starvation period yielded better results with *Tetrahymena* spp. used in their chemotactic test; some of our preliminary data indicate a similar trend;

B. Exploration of the reasons underlying test variability and the provision of some possible solutions;

C. Implementation of the test with other ciliate species (other than *Tetrahymena vorax*):

some species may be more sensitive or more predictable than others in a test such as this one; as a result, several other species to be tested might include (but should not be limited to): *Paramecium spp., Tetrahymena corlissi, T. thermophila, Colpidium campylum, Glaucoma chattoni.*

D. Calibration of the test through an interlaboratory comparison:

in order to evaluate the reproducibility, clarity and comprehensibility of the test protocol, different regulatory laboratories should be sent a complete package with known reference toxicants and the essentials for running a test and results should be compared (this has been done for other test protocols and is important for ensuring the rigour of the protocol, e.g., see Kovacs and Ferguson 1990);

E. Variation of the exposure time:

vary from 15 minutes since other assays run for slightly longer; this may be necessary for detectable responses for some toxicants.

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Table 1. Results from Control Test Runs * using the TMTA.

Maze Number	n	Mean I_{tox}
1	1	0.46
2	3	0.49
3	4	0.49
6	3	0.45
8	3	0.46
9	1	0.55
11	1	0.56
12	5	0.47
13	2	0.60
14	3	0.63
15	1	0.48
17	4	0.55
18	2	0.51
20	1	0.47
21	2	0.45
22	1	0.49
23	7	0.49
24	2	0.45
U **	10	0.50
Mean	56	0.50

* dilution water in both arms; no toxicants

** unidentified mazes

Table 2. Results from Control * Test Runs, TMTA: Intramaze variability.

Maze Number	n	s **	Mean I_{tox}	CV ***
2	3	.06	0.49	11.62
3	4	.05	0.49	10.09
6	3	.09	0.45	20.93
8	3	.09	0.46	19.28
12	5	.05	0.47	11.32
13	2	.02	0.60	3.33
14	3	.17	0.63	27.57
17	4	.05	0.55	8.26
18	2	.09	0.51	16.83
21	2	.09	0.45	19.10
23	7	.07	0.49	14.94
24	2	.03	0.45	5.62
Mean				14.07

* dilution water in both arms; no toxicants

** standard deviation

*** coefficient of variation = $(s/\bar{X}) * 100$

Table 3. Summary of Reference Toxicant and Effluent Test Runs with the TMTA protocol.

	Number of runs	
Reference Toxicants		
Zinc	4	
Sodium Chloride	11	
4-chlorophenol	7	
Cadmium	3	
TOTAL	25	
Effluents		
	Number of effluents	Number of runs/effluent
Pulp and Paper sector		
Company 1	4	3
Company 2	1	3
Company 3	1	3
Company 4	1	3
Metal Casting sector		
Company 5	2	3
Organic sector		
Company 6	1	3
Iron and Steel sector		
Company 7	1	3
TOTAL	11	33

Table 4. Toxicity of standard reference toxicants to ciliated protists.

Compound	Effective Concentration	Species*/Method	Reference
Zinc			
	0.1-0.5 mg l^{-1}(min)	T.p./oxygen uptake	Slabbert and Morgan (1982)
	100 mg l^{-1} (min)	C.c./growth inhib.	Dive and Leclerc (1975)
	No effect response	T.v./toxitaxis	This study
Sodium chloride			
	2500 mg l^{-1} (LOEC)	T.v./toxitaxis	This study
4-Chlorophenol			
	No effect response	T.v./toxitaxis	This study
Cadmium			
	0.1-0.5 mg l^{-1}(min)	T.p./oxygen uptake	Slabbert and Morgan (1982)
	0.2 mg l^{-1} (min)	C.c./growth inhib.	Dive and Leclerc (1975)
	0.35-0.7 mg l^{-1}	T.p./chemotaxis	Berk *et al.* (1985)
	2.6 mg l^{-1}	T.p./growth inhib.	Houba *et al.* (1981)
	0.092 mg l^{-1} (LC_{50})	T.p./chemotaxis	Roberts and Berk (1990)
	No effect response	T.v./toxitaxis	This study

* Species Legend
T.p. = *Tetrahymena pyriformis*; C.c. = *Colpidium campylum*; T.v. = *Tetrahymena vorax*

Table 5. Toxicity of standard reference toxicants to Daphnia magna

Compound	Effective Concentration	Reference
Zinc		
	0.56 mg l^{-1}	Khangharot and Ray (1987)
	0.45-0.65 mg l^{-1}	B.A.R. Environmental (unpubl. data)
	0.56-1.3 mg l^{-1}	OMOE (unpubl. data)
	0.56-1.7 mg l^{-1}	Berglind and Dave (1984) Elnabarawy *et al.* (1986)
Sodium chloride		
	3536-4360 mg l^{-1}	B.A.R. Environmental (unpubl. data)
	3200-6300 mg l^{-1}	OMOE (unpubl. data)
	2250-4500 mg l^{-1}	Keating and Dagbuson (1986)
	1661-4571 mg l^{-1}	Cowgill (1987)
4-Chlorophenol		
	3.82 mg l^{-1}	B.A.R. Environmental (unpubl. data)
	2.5-7.3 mg l^{-1}	OMOE (unpubl. data)
Cadmium		
	0.6 mg l^{-1}	Bringmann and Kuhn (1977)
	0.142-0.267 mg l^{-1}	OMOE (unpubl. data)

Table 6. Toxicity of standard reference toxicants to Rainbow trout

Compound	Effective Concentration	Reference
Zinc		
	0.55 mg l^{-1}	Hale (1977)
	0.16 mg l^{-1}	Bradley and Sprague (1985)
	0.40-1.0 mg l^{-1}	B.A.R. Environmental (unpubl. data)
Sodium chloride		
	Not available	
4-Chlorophenol		
	0.15-0.18 mg l^{-1}	OMOE (unpubl. data)
Cadmium		
	2.5-27 mg l^{-1}	Environment Canada (1990)

Table 7. Results of Microplate LC_{50} Tests with Zinc using Tetrahymena vorax

	Percentage Mortality		
Zn conc. (ppm)	Prep 1	Prep 2	Prep 3
0	-21.2	24.3	2.4
0.107	15.4	18.3	19.0
0.214	44.2	11.9	33.3
0.429	50.0	9.6	38.1
0.857	40.4	27.5	46.4
1.714	45.2	39.4	50.0

Table 8. Comparison of TMTA LOEC results with Daphnia magna and Rainbow trout LC_{50} results with the same effluent samples.

Sample Number	Industrial sector	TMTA LOEC*	DM LC_{50}**	RT LC_{50}***
03910104	Pulp and Paper	50.0 %	35.8 %	16.6 %
03910155	"	100 %	61.4 %	12.63 %
03910020	"	NS	53.2 %	46.7 %
03910223	"	50.0 %	50.9 %	16.61 %
03910178	Metal Casting	NS	18 %	80.6 %
03910095	"	NS	0.19 %	0.65 %
03910073	Organic	NS	16.2 %	51 %
03910110	Pulp and Paper	NS	> 100 %	NL
03910115	Pulp and Paper	NS	68.4 %	36.3 %
03910114	Pulp and Paper	NS	36.7 %	NL
03910044	Iron and Steel	NS	19.9 %	16.9 %

* The T-Maze Toxitactic Assay with LOEC value (this study)

** *Daphnia magna* 48-hour LC_{50} (Poirier *et al.* 1988)

*** Rainbow trout 96-hour LC_{50} (Craig *et al.* 1983)

NS = No sublethal response

NL = No lethal response

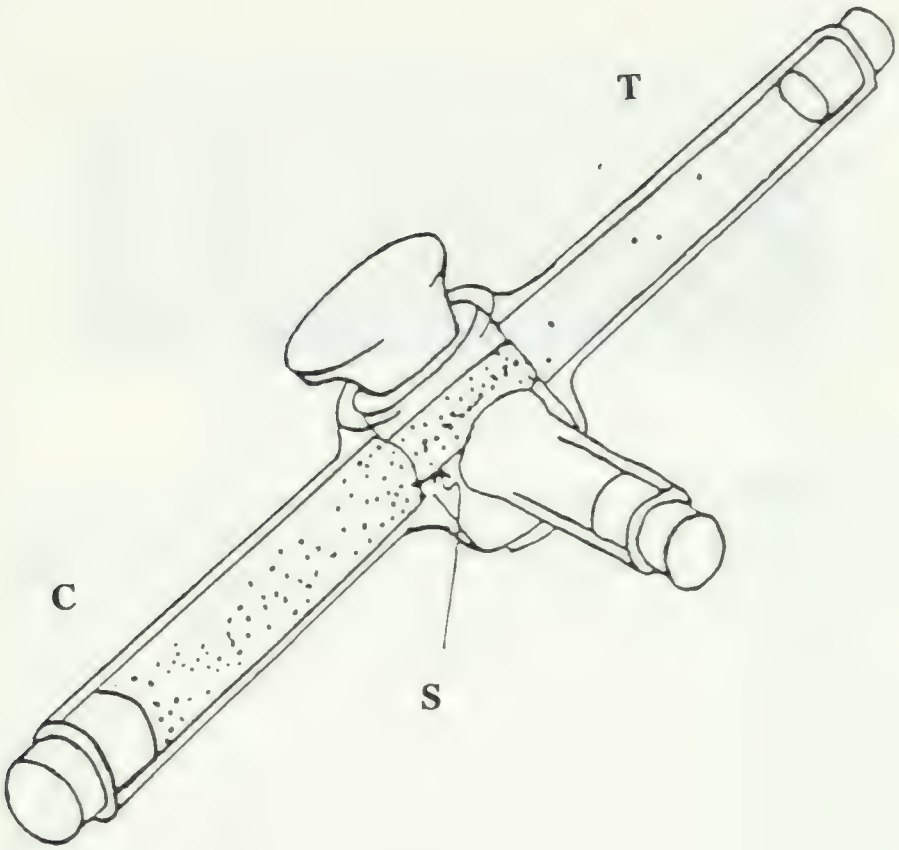

Figure 1. T-maze configuration for the TMTA with Tetrahymena vorax. Cells in control solution fill the stopcock barrel (S); control solution fills C arm; test solution fills T arm. Assay begins when bore is turned to connect the two arms. Cells distribute for an exposure period of 15 minutes at which time the stopcock is turned, arms emptied and cells enumerated. Index of toxitaxis (I_{tox}) is calculated as above (modified from Van Houten et al. 1982).

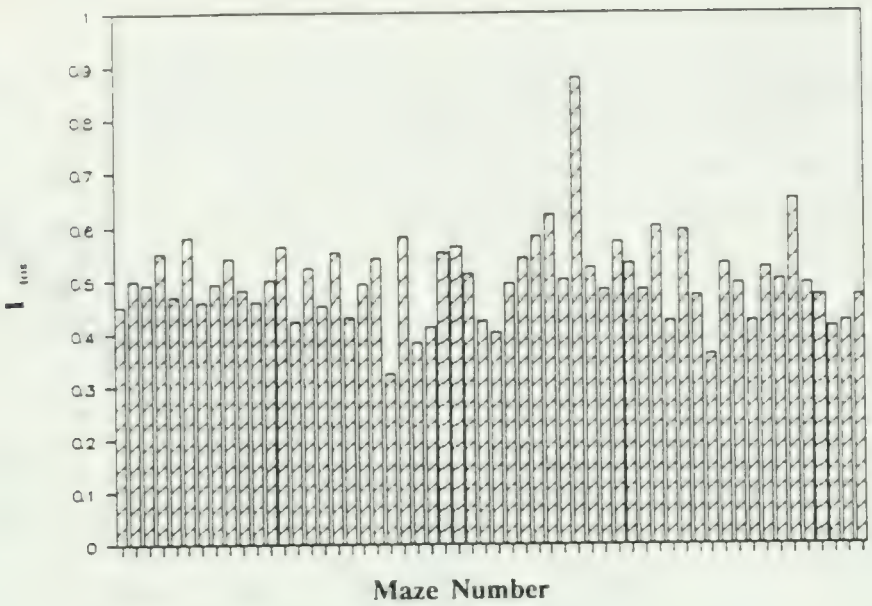

Figure 2. Results of all control maze tests for the TMTA protocol.

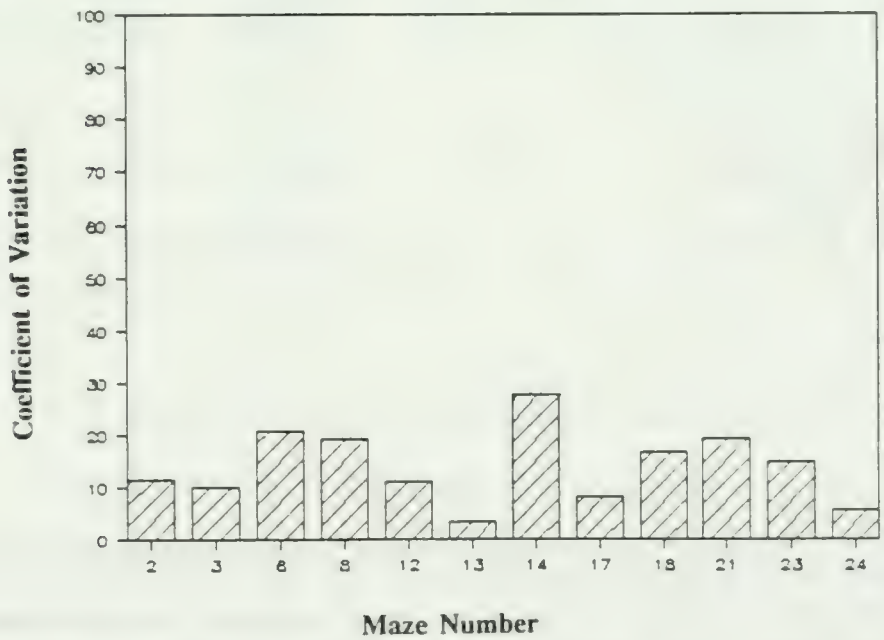

Figure 3. Intramaze variability for control maze tests using the TMTA protocol.

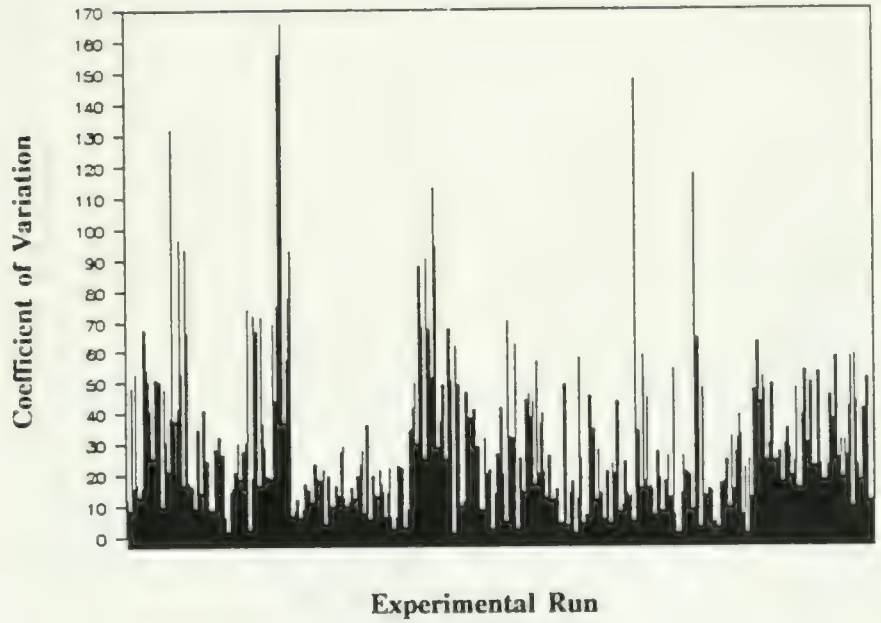

Figure 4. **Results from cell enumeration precision data for all experimental runs using the TMTA protocol.**

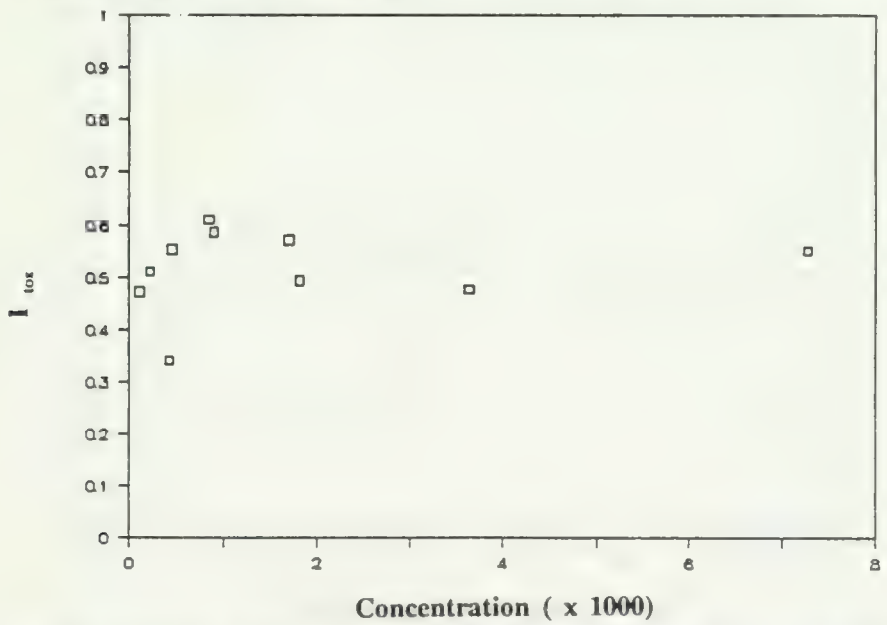

Figure 5. **Results from reference toxicant tests for zinc using the TMTA protocol.**

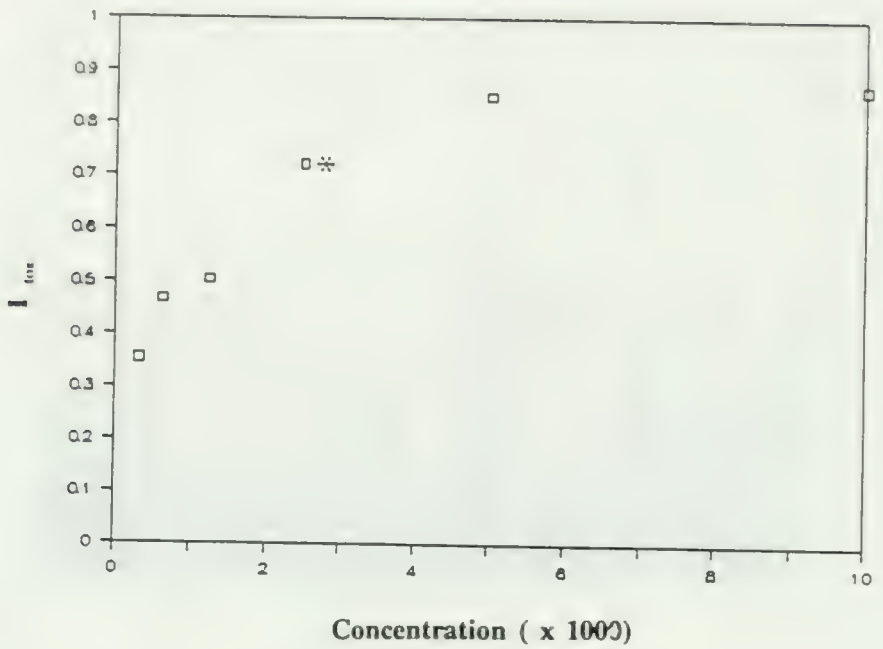

Figure 6. **Results from reference toxicant tests for sodium chloride using the TMTA protocol (* = LOEC).**

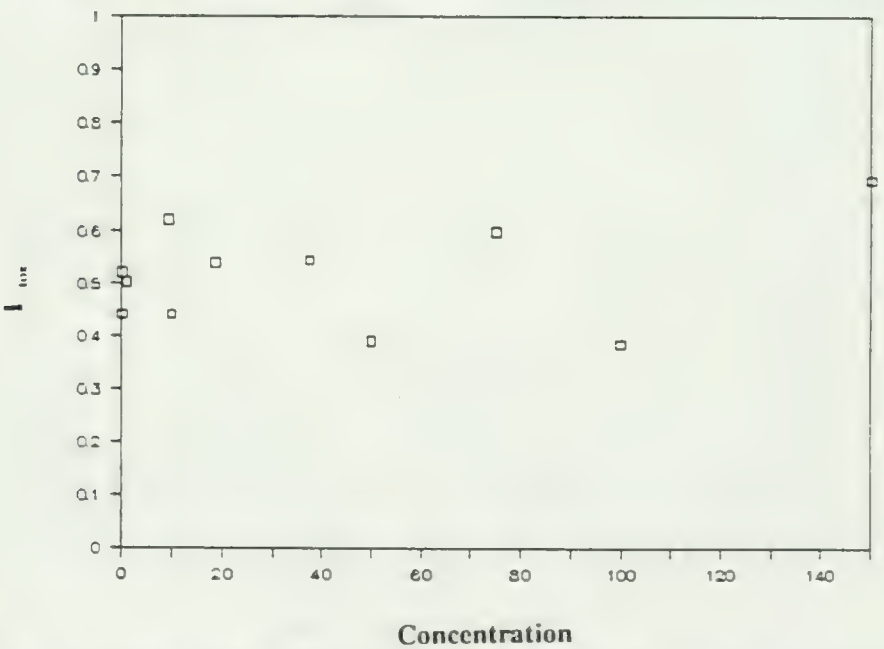

Figure 7. **Results from reference toxicant tests for 4-chlorophenol using the TMTA protocol.**

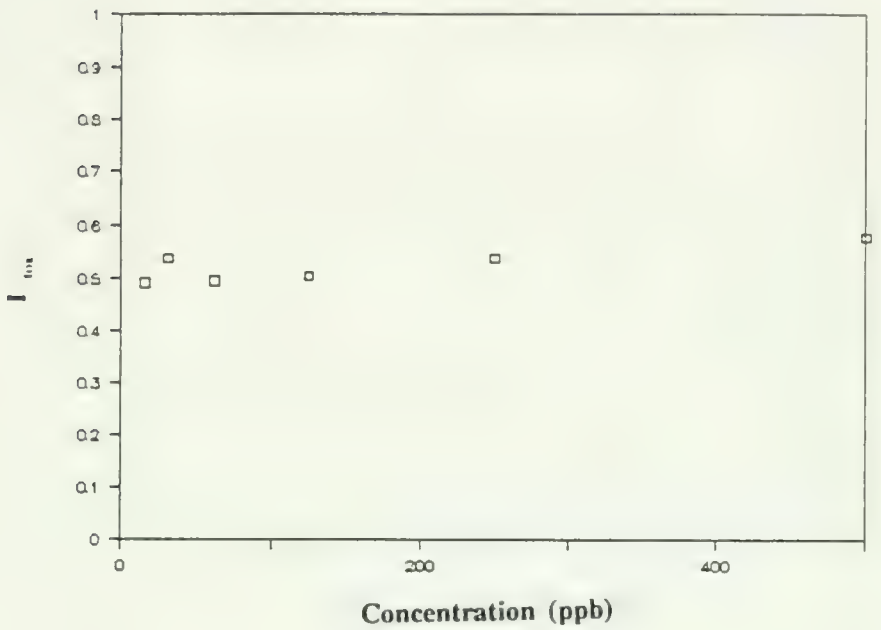

Figure 8. **Results from reference toxicant tests for cadmium using the TMTA protocol.**

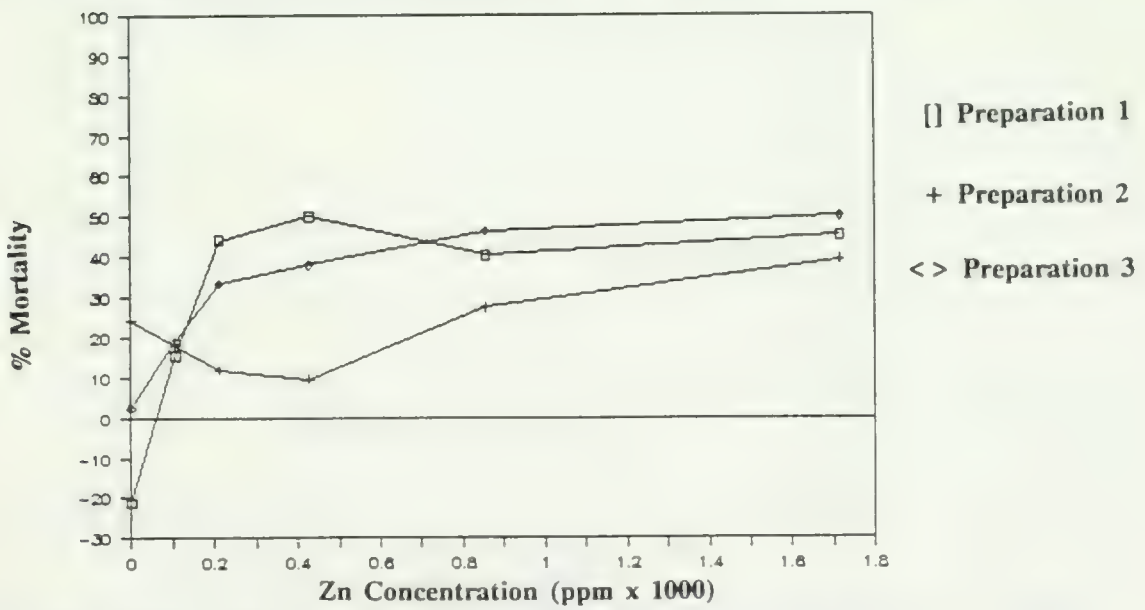

Figure 9. **Results from Tetrahymena vorax microplate LC_{50} tests with zinc.**

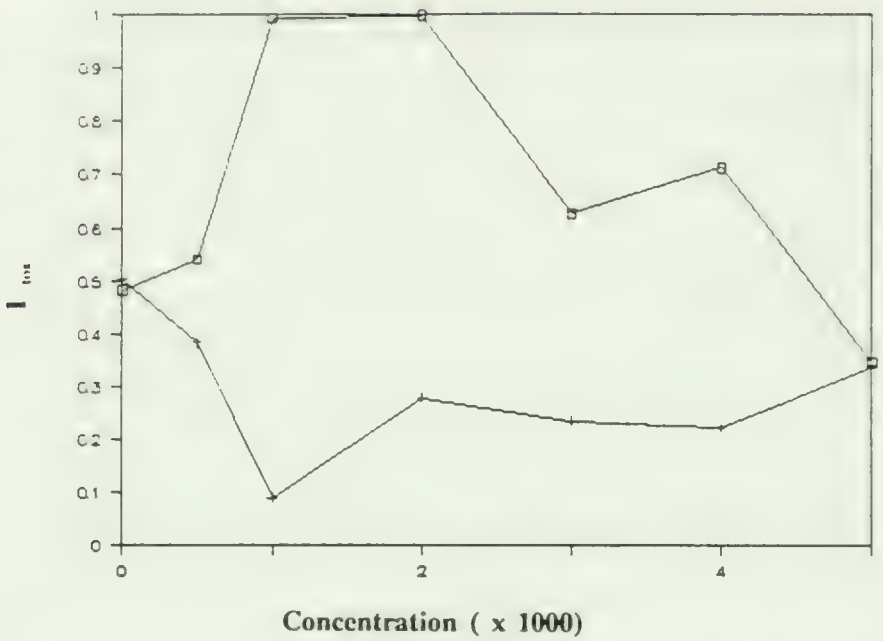

Figure 10. Results from reference toxicant tests for sodium chloride and zinc using the TMTA protocol (with pre-treatment with MOPS starvation buffer medium (Tetrahymena vorax)).

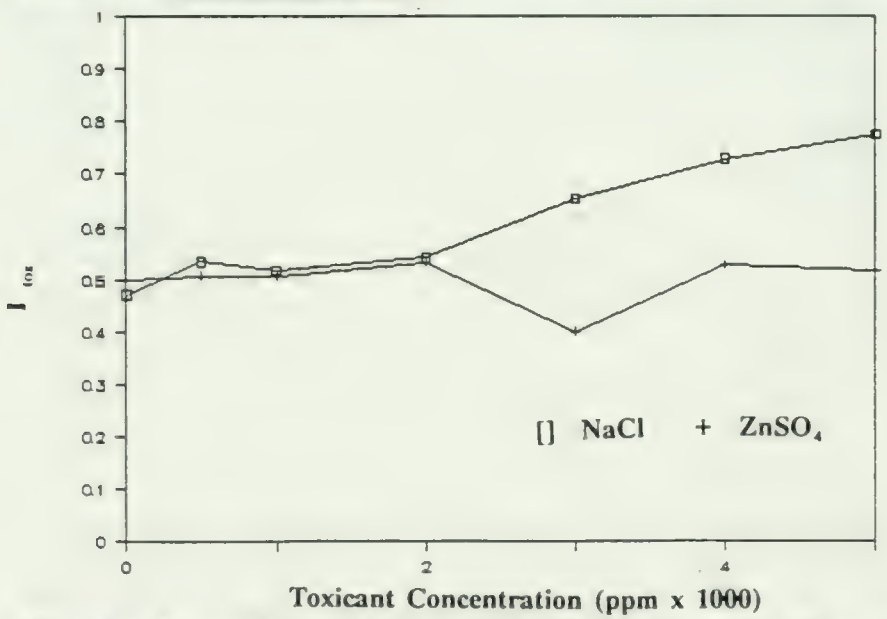

Figure 11. Results from reference toxicant tests for sodium chloride and zinc using the TMTA protocol (with pre-treatment with MOPS starvation buffer medium (Tetrahymena thermophila)).

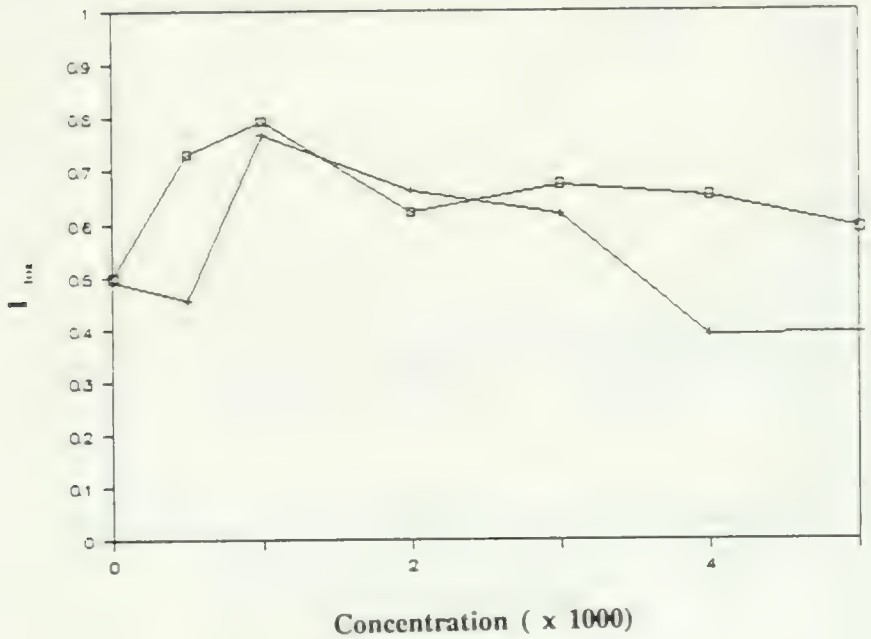

Figure 12. **Results from reference toxicant tests for sodium chloride and zinc using the TMTA protocol (with pre-treatment with MOPS starvation buffer medium (Glaucoma chattoni)).**

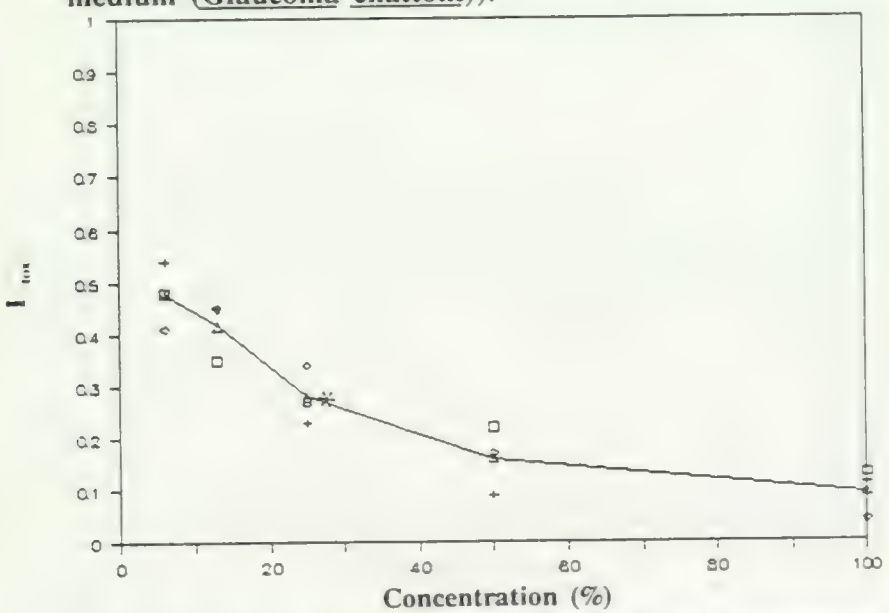

Figure 13. **Results for pulp and paper effluent sample # 03910104 using the TMTA protocol (* = LOEC).**

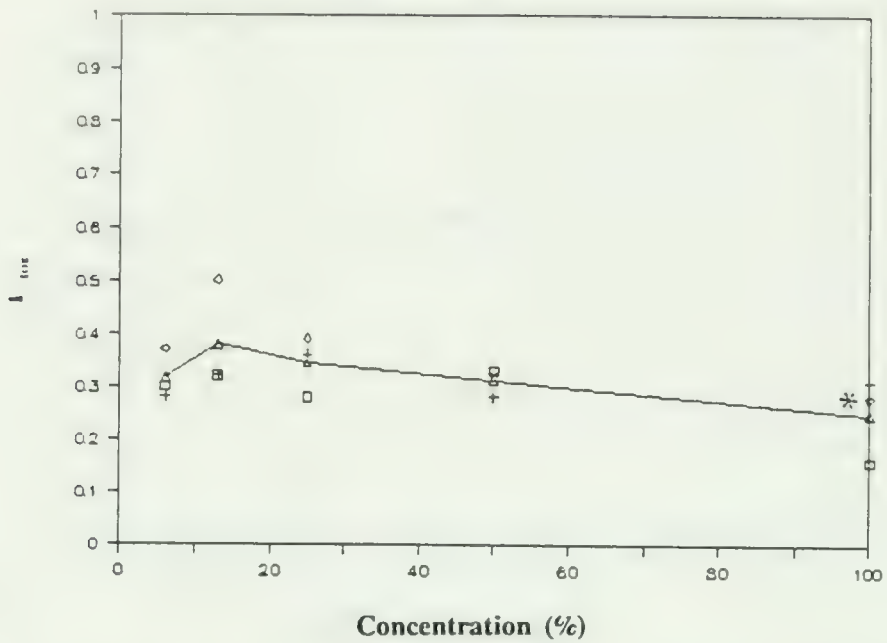

Figure 14. Results for pulp and paper effluent sample # 03910155 using the TMTA protocol (* = LOEC).

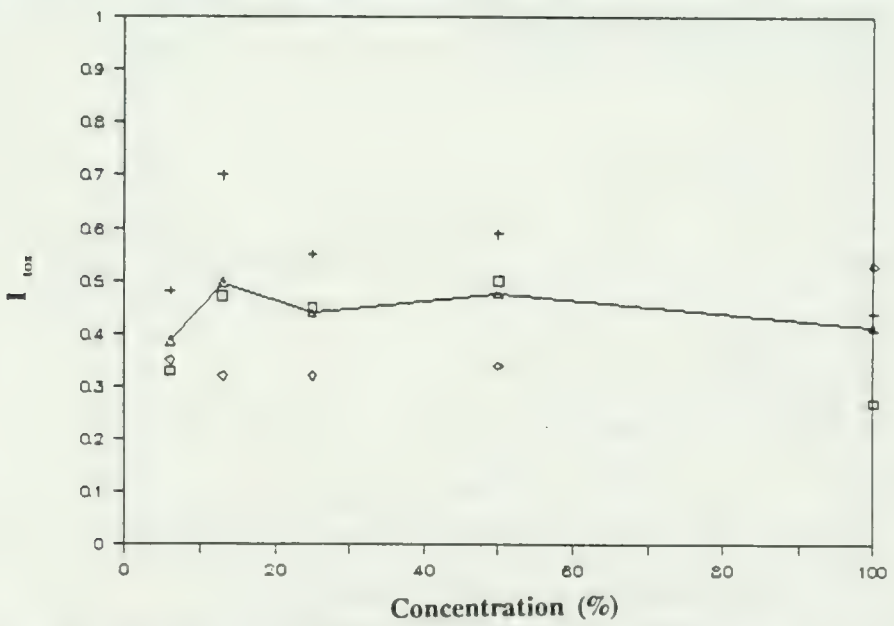

Figure 15. Results for pulp and paper effluent sample # 03910020 using the TMTA protocol.

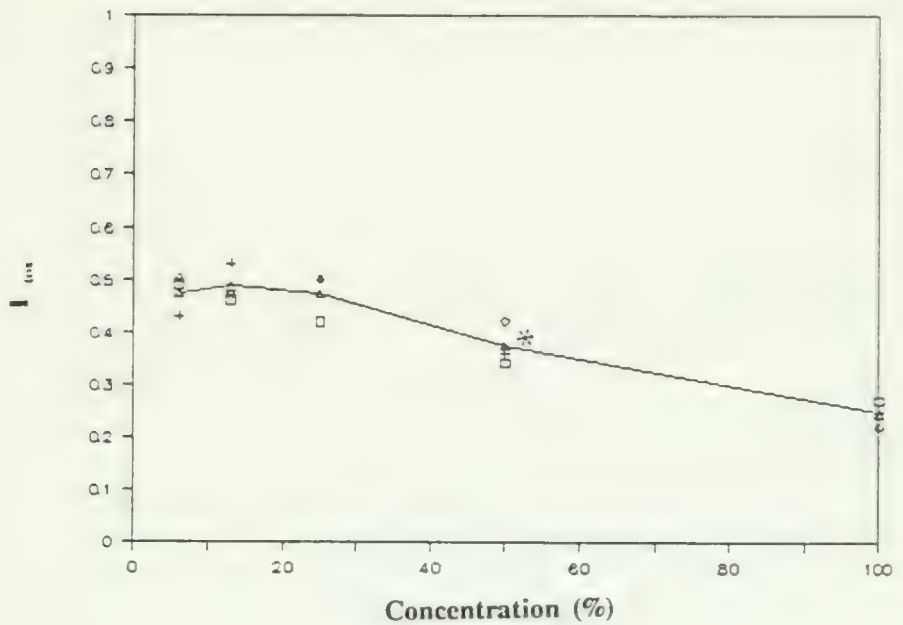

Figure 16. **Results for pulp and paper effluent sample # 03910223 using the TMTA protocol (* = LOEC).**

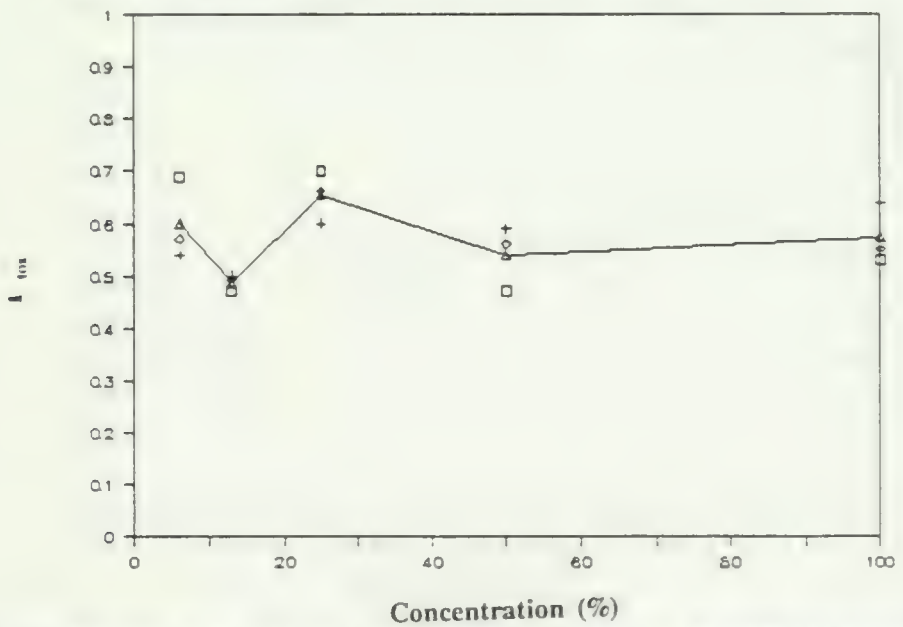

Figure 17. **Results for pulp and paper effluent sample # 03910110 using the TMTA protocol.**

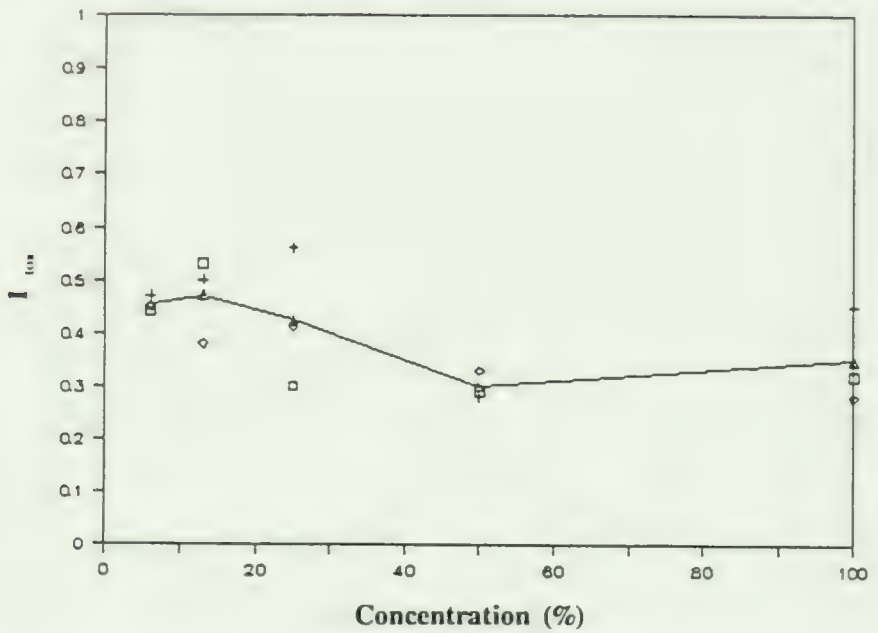

Figure 18. Results for pulp and paper effluent sample # 03910115 using the TMTA protocol.

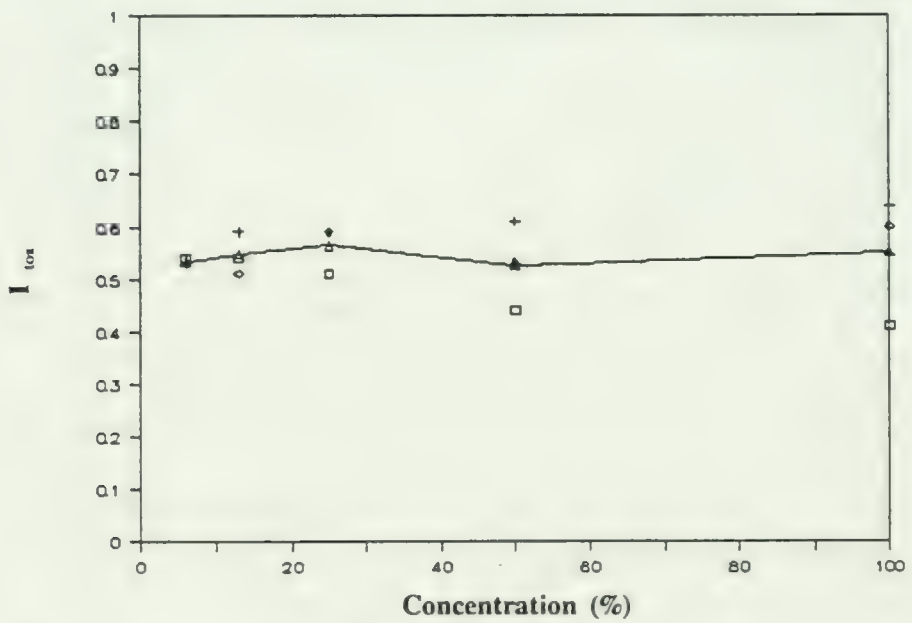

Figure 19. Results for pulp and paper effluent sample # 03910114 using the TMTA protocol.

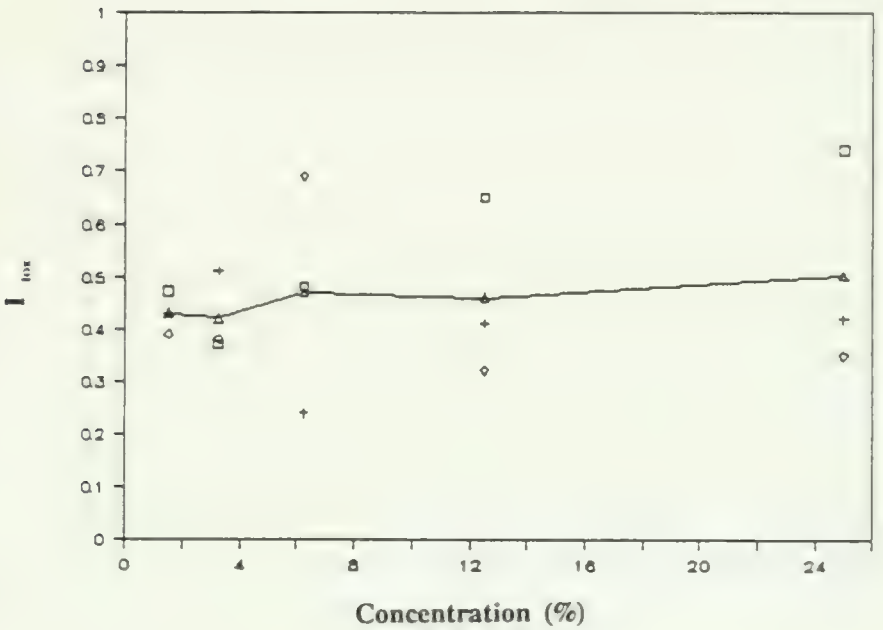

Figure 20. **Results for metal casting effluent sample # 03910178 using the TMTA protocol.**

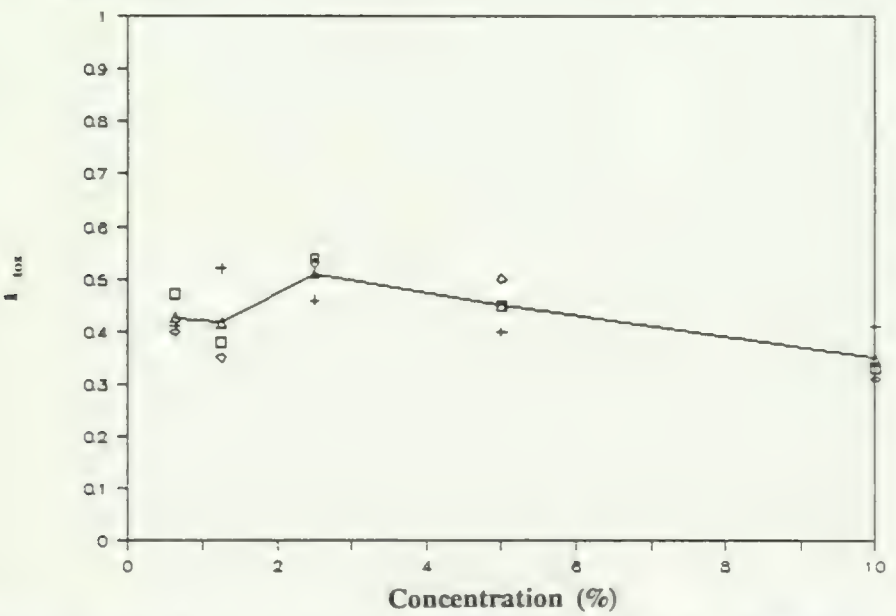

Figure 21. **Results for metal casting effluent sample # 03910095 using the TMTA protocol.**

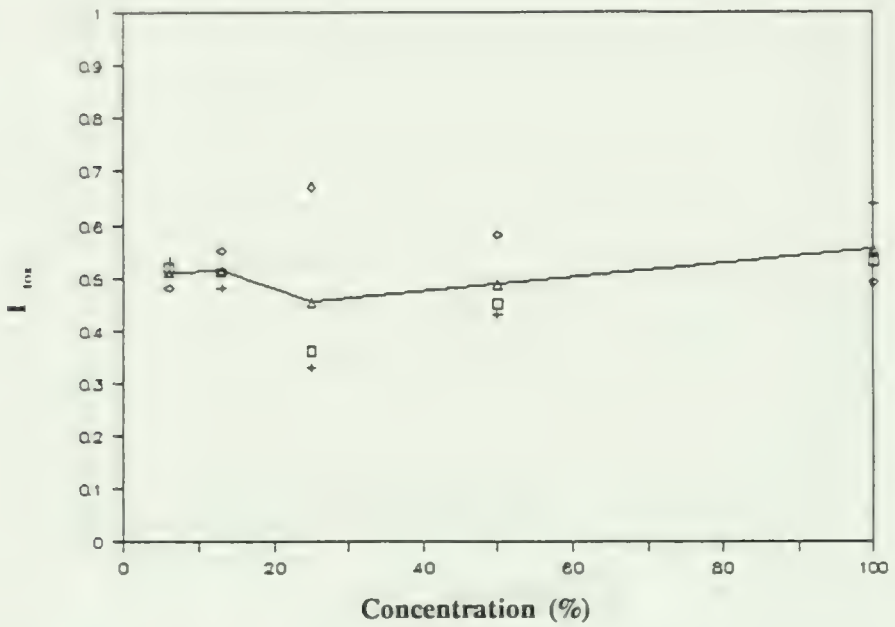

Figure 22. **Results for organic effluent sample # 03910073 using the TMTA protocol.**

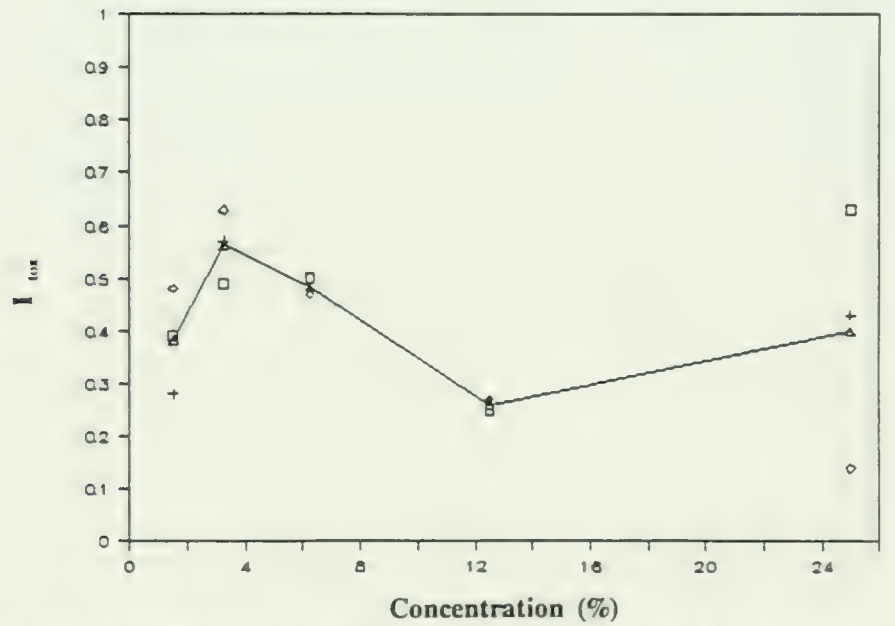

Figure 23. **Results for iron and steel effluent sample # 03910044 using the TMTA protocol.**

APPENDICES

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Appendix I - List of equipment parts used in the T-Maze Toxitactic Assay (TMTA) protocol

Part	Supplier	Catalogue Number
Stopcock	Lab Glass*	#LG9680106S
T-maze apparatus	Lab Glass*	#LG9601-114
Corks	Canlab**	#C8215-2TC
Palmer counting cell	Thomas Scientific***	#9853-N10

* Address: Lab Glass Inc., P.O. Box 610, 1172 NorthWest Blvd., Vineland, NJ, USA 08360. Telephone: (609) 691-3200

** Address: AHS/Canlab, 2390 Argentia Road, Mississauga, Ontario, CANADA L5N 3P1

*** Address: Thomas Scientific, 99 High Hill Road, P.O. Box 99, Swedesboro, NJ, USA 08085. Telephone: (609) 467-2000

Appendix II - Physico-chemical description of laboratory dilution water used in the TMTA protocol

Parameter	Value (mg l^{-1})
pH	8.5
Conductivity (umho)	252
TSS	< 1
Chromium (VI)	< 0.01
COD	13
TOC - Beckmann	10
DOC - Beckmann	10
Total Cyanide	0.001
Free Cyanide	0.001
Ammonia (an N)	0.032
TKN (as N)	< 0.2
Hardness (as $CaCO_3$)	161
Fluoride	0.27
Chloride	0.76
Nitrite (as N)	< 0.04
Bromide	< 0.8
Nitrate (as N)	< 0.04
Phosphate (as P)	< 0.8
Sulfate	17
Alkalinity (as $CaCO_3$)	150
Antimony	< 0.005
Arsenic	0.014
Mercury	< 0.0001
Selenium	< 0.005
Calcium	35
Magnesium	18
Sodium	4
Potassium	0.95
Aluminum	0.071
Barium	0.056
Beryllium	< 0.001
Boron	0.21
Cadmium	< 0.002
Chromium	< 0.004
Cobalt	< 0.01
Copper	< 0.006
Iron	0.073
Lead	< 0.02
Manganese	0.007
Molybdenum	< 0.02
Nickel	< 0.01
Phosphorus	< 0.06
Silicon	5.4
Silver	< 0.01
Strontium	0.094
Sulfur	7.4
Thallium	< 0.06
Titanium	< 0.01
Vanadium	< 0.005
Zinc	0.015
Zirconium	< 0.01

Appendix III - Standard data bench sheet for the T-Maze Toxitactic Assay (TMTA) protocol

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BAR. SAMPLE NO _____

Tetrahymena Toxitactic Bioassay Bench Sheet

Basic Information

Industry _____
Location _____
Substance _____
IMIS _____ Date Rec'd. _____
Pipe _____ Date Tested _____
Sample Method _____ Time Started _____
Name Coll _____ Shipped by _____
Date Coll _____ Storage _____
Age of Culture _____ Volume counted _____
Other comments _____

Toxitactic Bioassay Data Sheet

Conc.		Count 1	Count 2	Count 3	X	I tox	pH	DO mg/L	Cond	Temp
	T									
	C									
	T									
	C									
	T									
	C									
	T									
	C									
	T									
	C									
	T									
	C									
	T									
	C									
	T									
	C									
	T									
	C									

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Appendix IV - Standard data bench sheet for Tetrahymena vorax microplate 24-hour LC_{50} test

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24 HOUR LC50 FOR TETRAHYMENA VORAX

Age of cells _____

Substance tested _____

Name collected _____

Date collected _____

Date and time set _____

TEST REPLICATE NUMBER _____

CONCENTRATION _________

Time = 0 hours		Time = 24 hours	
Well ID	Count	Well ID	Count
Average		Average	

CONCENTRATION _________

Time = 0 hours		Time = 24 hours	
Well ID	Count	Well ID	Count
Average		Average	

CONCENTRATION _________

Time = 0 hours		Time = 24 hours	
Well ID	Count	Well ID	Count
Average		Average	

CONCENTRATION _________

Time = 0 hours		Time = 24 hours	
Well ID	Count	Well ID	Count
Average		Average	

CONCENTRATION _________

Time = 0 hours		Time = 24 hours	
Well ID	Count	Well ID	Count
Average		Average	

CONCENTRATION _________

Time = 0 hours		Time = 24 hours	
Well ID	Count	Well ID	Count
Average		Average	

Distribution List

All District and Regional Offices (MOE)
Hazardous Contaminants Branch
Water Resources Branch

University Libraries
College Libraries (Canadore, Fanshaw, Seneca, Mohawk, Georgian, Centennial)
Private Consultants (BEAK, BAR Environmental, Pollutech, Integrated Explorations, Lakefield Research, AECL (Manitoba), Ontario Hydro, Domtar (Quebec), SENTAR ENVIRONMENTAL CONSULTANTS (B.C.))

Environment Canada (Conservation and Protection - Ottawa)